Clot-derived contaminants in transplanted bone marrow mononuclear cells impair the therapeutic effect in stroke

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Background and Purpose: The beneficial effects of bone marrow mononuclear cell (BM-MNC) transplantation in preclinical experimental stroke have been reliably demonstrated. However, only overall modest effects in clinical trials were observed. We have investigated and report a cause of the discrepancy between the pre-clinical and clinical studies.

Methods: To investigate the possible cause of low efficacy of BM-MNC transplantation in experimental stroke, we have focused on blood clot formation which is not uncommon in human bone marrow aspirates. In order to evaluate the effects of clot-derived contaminants in transplanted BM-MNC on stroke outcome, a murine stroke model was employed.

Results: We show that BM-MNC separated by an automatic cell isolator (Sepax2), which does not have the ability to remove clots, did not attenuate brain atrophy after stroke. In contrast, manually isolated, clot-free BM-MNC exerted therapeutic effects. Clot-derived contaminants were also transplanted intravenously to post-stroke mice. We found that the transplanted contaminants were trapped at the peri-stroke area, which were associated with microglial/macrophage activation.

Conclusion: Clot-derived contaminants in transplanted BM-MNC nullify therapeutic effects in experimental stroke. This may explain neutral results in clinical trials, especially in those using automated stem cell separators that lack the ability to remove clot-derived contaminants.

Introduction

The beneficial effects of bone marrow mononuclear cells (BM-MNC) transplantation in experimental stroke model had been shown in a number of experiments.[1,2] BM-MNC contain various cells, including lymphoid, myeloid, erythroid, and stem cell populations. Although individual BM-MNC populations were shown to exert different effects on stroke recovery[3], multiple therapeutic BM-MNC effects have also been proposed, including neuroprotection by monocytes within the myeloid fraction[3,4], and induction of neurogenesis by CD34+ hematopoietic stem/progenitor cells (HSC)[5] representing about 1.5 % of BM-MNC.[6] Clinical trials employing BM-MNC transplantation for stroke have been conducted and both positive[6] and neutral[7,8] results have been reported. Mixed results were also reported after BM-MNC transplantation in myocardial infarction.[9] Meta-analysis of clinical trials have revealed the safety and feasibility of the approach but further well-designed large-scale, randomized and controlled trials are required to investigate their efficacy.[10] The discrepancy between the positive effects in preclinical studies and the overall modest effects in clinical trials led us to investigate the role of an inhibitor which suppresses therapeutic effects of BM-MNC in clinical trial.

Human bone marrow aspirate is prone to clot formation due to its high viscosity[11] and the presence of lipids, connective tissue, and megakaryocytes.[12] Damaged cells and substances from these can activate microglia/macrophages, causing proinflammatory responses[13] which

can have detrimental effects on post-stroke recovery.[14] Based on these findings, we have investigated the effect of clot-derived contaminants in BM-MNC transplantation after cerebral ischemia in experimental stroke model.

Methods

This article adheres to the American Heart Association Journals Implementation of the Transparency and Openness Promotion Guidelines. The data supporting the findings of this study are available from the corresponding author on reasonable request.

All animal experiments were approved by the Animal Care and Use Committee of Foundation for Biomedical Research and Innovation and complies with the Guide for the Care and Use of Animals published by the Japanese Ministry of Education, Culture, Sports, Science and Technology.

Murine stroke model

Seven weeks old male severe combined immunodeficiency CB-17/lcr-scid/scidJcl (SCID) mice (Oriental Yeast, Tokyo, Japan) were used as recipients for human BM-MNC. Wild type 7 weeks old male CB-17/lcr-+/+Jcl mice (Oriental Yeast) received litter mate murine BM-MNC. Experimental design is shown in Figure 1. Permanent middle cerebral artery occlusion (MCAO) was induced as described previously.[5,15] Briefly, the distal MCA portion was ligated

and disconnected using bipolar forceps under 3 % isoflurane inhalation anesthesia. During surgery, rectal temperature was monitored and maintained at 37.0±0.2°C using a feedback-regulated heating pad. Cerebral blood flow (CBF) in the MCA territory was measured with 2D laser blood flow imaging (Muromachi, Tokyo, Japan). All mice showed a ~75 % decreased CBF immediately after MCA occlusion as well as 48 hr after ligation.

Preparation of human BM-MNC

Human bone marrow (BM) aspirate was purchased from LONZA (Basel, Switzerland) and BM-MNC were purified manually or by an automatic cell separator (Sepax2; GE Healthcare, Buckinghamshire, UK) at day 4 after harvest. All materials and reagents, including human BM aspirate and density centrifugation medium (Ficoll-Paque Premium, GE) were identical between the procedures. For manual separation, BM was diluted 4-fold with saline (Otsuka Pharmaceutical, Tokyo, Japan), layered on Ficoll-Paque Premium, and centrifuged at 400*g* for 40 min. Cells in buffy coat, whilst carefully avoiding any clot contamination, were isolated manually and washed 3 times with saline. Clots in buffy coat were also collected and washed 3 times with saline and digested using 23G needle/syringe. For automated purification, 50 ml of human BM was loaded to Sepax2, and BM-MNC were isolated with Ficoll-Paque Premium using the automatic cell separation mode according to manufacturer's instruction. The number of white blood cells (WBC), RBC and platelets/cell debris in randomly selected 0.01 mm^2 were

counted a by blinded investigator (N=6, in each samples).

Murine BM-MNC preparation

Murine BM was obtained from tibias and femurs of adult male CB-17 mice, resuspended by saline, layered on Ficoll-Paque Premium, and centrifuged at 400*g* for 40 min. BM-MNC in buffy coat were isolated manually. For clot formation, thrombin (50 U final concentration, Mochida, Tokyo, Japan), Alexa Fluor 647-labelled fibrinogen (0.6 mg/ml final concentration, Molecular Probes, OR, USA), and $CaCl_2$ (5 mM in final concentration) were added to BM-MNC and incubated for 30 s at room temperature. For experiment using labelled cells, BM-MNC were stained with green fluorescent dye (Cell tracker Green CMFDA, 5 μM final concentration, Molecular Probes, OR, USA) before clot formation. Clots were washed twice with PBS, digested using 23G needle/syringe, and filtered through cell strainers (pore size 35 μm, BD Bioscience, MA, USA) before injection.

Cell transplantation

In each experiment, $1x10^5$ BM-MNC in 100 μl PBS or vehicle was injected intravenously 48 h after stroke induction. For morphological and immunohistochemical analysis, mice were anesthetized with sodium pentobarbital, and perfused transcardially with saline followed by 4% paraformaldehyde. Whole brain images were captured by a digital camera system

(Olympus, Tokyo, Japan), and the area of each hemisphere was measured with ImageJ (National Institutes of Health, Bethesda, MD, USA) by blinded investigators. The value of [(right hemisphere) – (left hemisphere)]/(right hemisphere) x 100 was defined as brain atrophy in area (%).

Immunohistochemistry

Coronal sections (20 μm) were prepared using a vibratome (Leica,Wetzlar, Germany) and immunostained with primary antibodies against F4/80 (dilution 1:50; Serotec, Raleigh, NC, USA), CD11b (dilution 1:250; Serotec, Raleigh, NC, USA), or CD31 (dilution 1:50; BD Pharmingen, San Jose, CA, USA). Anti-CD11b, and anti-CD31 were visualized by the 3,3'-diaminobenzidine method, and counterstained with Mayer's hematoxylin solution (Wako, Osaka, Japan). The number of CD11b positive cells in a randomly selected 0.1 mm^2 region of interest in the peri-stroke area was quantified by a blinded investigator. To assess the deposition of clot-derived cell debris and fibrinogen at microglia/macrophages, Alexa555-coupled antibodies (dilution 1:500; Thermo Fisher Scientific, Waltham, MA, USA) were used as the secondary antibody to anti-F4/80.

Cell analysis by fluorescence-activated cell sorting (FACS)

Anti-CD45 (PE, BD Bioscience) and Ter119 (PerCP-Cy5.5, BD Bioscience) antibodies were

used to identify WBC and RBC, respectively. Cells were incubated with antibodies for 15 min at 4°C and washed with PBS containing 1 % of fetal bovine serum (Thermo Fisher Scientific). A FACS Calibur (BD Bioscience) was used for cell analysis. CD45-positive cells with reduced FSC signal (outside of normal WBC gate) were considered as denatured WBC, and Ter119-positive cells with reduced FSC signal were counted as denatured RBC.

Data analysis

Statistical comparisons among groups were conducted using one-way analysis of variance (ANOVA) followed by post-hoc analysis using Dunnett's test. In all experiments, the mean ± SD is reported.

Results

Human BM-MNC isolated by Sepax2 did not attenuate post-stroke brain atrophy in murine stroke model

Sepax2 is a fit-for-purpose device for isolating BM-MNC by gravity centrifugation and is broadly used clinically for allogenic hematopoietic stem cell transplantation.[16] However, Sepax2 does not have the ability to remove clots from the buffy coat. We compared the effect of Sepax2 and manually purified BM-MNC on experimental stroke outcomes.

Figure 2A shows BM-MNC and clots at the buffy coat after gravity centrifugation as applied

during manual cell isolation. It should be noted that most RBC precipitate at the bottom, but small clots remain within the buffy coat after centrifugation. It was carefully avoided to aspirate these clots during manual processing. Figure 2B shows manually isolated BM-MNC, Sepax2-isolated BM-MNC, and clots at the buffy coat derived cells, respectively. In contrast to manual separation, a number of RBC and platelets/cell debris were observed in Sepax2-isolated BM-MNC. RBC and platelets/cell debris were also observed in BM-MNC suspension obtained from clots at the buffy coat. Figure 2C shows the ratio of contaminated RBC and platelet/cell debris versus WBC in each sample, revealing significantly higher contamination in Sepax2-isolated BM-MNC compared to manually isolated BM-MNC.

We had previously demonstrated that CD34+ HSC transplantation after experimental stroke induces regenerative process and attenuates of post-stroke brain atrophy.[5] Here, Sepax2-isolated, clot-derived or manually isolated BM-MNC were intravenously injected into stroke mice 48 h after stroke induction. At day 30 after cell transplantation, whole brain images were captured to investigate the effect of cell transplantation (Figure 3A). The attenuation of the atrophy was evaluated and we found that mice receiving manually separated BM-MNC showed attenuated post-stroke brain atrophy compared to PBS controls (Figure 3B). In contrast, mice which received BM-MNC isolated by Sepax2 showed no attenuation of brain atrophy.

Transplantation of Sepax2-isolated BM-MNC preserves cerebral microvasculature but is

associated with increased CD11b-positive cell counts

We previously showed that BM-MNC transplantation after stroke preserves the microvascular structure followed by attenuation of brain atrophy and functional recovery.[17,18] To investigate the cause of the neutral results seen after Sepax2-isolated BM-MNC transplantation in clinical trials, brain tissue was harvested 24 h after cell transplantation to investigate effect on the microvasculature (Figure 4A). Compared with PBS-injected control mice, mice receiving manually isolated or Sepax2-isolated BM-MNC showed preservation of microvascular structures in the infarct area (Figure 4B).

We also showed that enhanced inflammatory responses within the peri-stroke area detrimentally affect stroke outcome.[18] To investigate the effect of cell transplantation on microglia/macrophage activation, brain sections were investigated using anti-CD11b antibodies at 24 h after cell transplantation. Compared with mice that received PBS or manually isolated BM-MNC, mice receiving Sepax2-isolated BM-MNC had higher numbers of CD11b-positive cells (microglia/macrophages) in peri-stroke areas (Figure 4C, D). These data indicate that cells isolated by Sepax2 can generally preserve microvasculature structures at infarct area but enhance inflammatory cell presence in peri-stroke areas.

Injection of clot-derived cells/debris enhances microglia/macrophage numbers in peri-stroke areas

To investigate the effect of clot-derived cells/debris on CD11b-positive microglia/macrophage numbers, clots were formed with mice BM-MNC, fibrinogen, thrombin and Ca^{2+} ions. Figure I (SUPPLEMENTAL INFORMATION) shows the results of FACS analysis before (Figure I, A-E) and after clot formation (Figure I, F-J). It should be noted that RBC were contaminated in isolated mice BM-MNC. The FSC level of CD45-positive WBC and Ter119-positive RBC (Figure I, I-J) was decreased after clot formation (Figure I, D and E, respectively). The percentage of degenerated WBC (*vs* all WBC-positive cells) was 3.4 %/ 48.4 % before/after clot formation. Similarly, the percentage of degenerated RBC (versus all WBC-positive cells) was 0.5 %/75.3 % before/after clot formation.

To confirm the hypothesis that clot-derived cells/debris interact with microglia/macrophages in peri-stroke areas, mice BM-MNC with contaminated RBC were labelled with CMFDA before clot formation. The BM-MNC before or after clot formation were intravenously transplanted 48h after stroke induction, and animals were sacrificed 20 minutes later. No transcardial perfusion with saline/paraformaldehyde was performed in this experiment to identify the localization of transplanted cells/debris. No microglia/macrophage was CMFDA- or Alexa 647-positive in mice that received cells before clot formation, and CMFDA and Alexa 647 signals were exclusively located inside the microvasculature (Figure 5A). In contrast, CMFDA and Alexa 647-positive microglia/macrophages were observed in mice that received clot-derived cells/debris (Figure 5B). These results indicate that clot-derived contaminants

interact microglia/macrophage in peri-stroke areas.

Removal of clot-derived denatured cells/debris restores BM-MNC the therapeutic effects

Compared to other density centrifugation medium, the advantage of Ficoll-paque had been suggested in comparison of clinical trials using BM-MNC transplantation for cardiac ischemia.[19] Ficoll-paque consists of Ficoll PM400 and sodium diatrizoate, and Ficoll PM400 is a highly branched hydrophilic sugar polymer[20-22] that can interact with denatured cells. Figure II (SUPPLEMENTAL INFORMATION) shows the FACS analysis of BM (not cells obtained from buffy coat, but freshly isolated cells from tibias and femurs) washed with PBS or Ficoll-PM400. The percentage of denatured WBC (vs all WBC-positive cell) was 10.0 % versus 5.9 % in cells washed with PBS and Ficoll-PM400, respectively. Similarly, the percentage of denatured RBC (versus all WBC-positive cell) was 3.3% versus 2.5% in cells washed with PBS (SUPPLEMENTAL INFORMATION, Figure II A-E) and Ficoll-PM400 (SUPPLEMENTAL INFORMATION, Figure II F-I), respectively. Cells were transplanted 48 h after stroke induction, and effects were investigated at day 30 after cell transplantation. Compared with PBS injected control, mice that received cells washed with Ficoll-PM400 showed significant attenuation of post-stroke brain atrophy at day 30, though cells washed with PBS failed to induce this effect (SUPPLEMENTAL INFORMATION, Figure II K). These results supported our hypothesis that removal of denatured WBC and RBC in transplanted BM-

MNC is essential for the cells' therapeutic effect after stroke.

Discussion

We report herein that clot-derived contaminants in transplanted BM-MNC impair the therapeutic effect, presumably be creasing proinflammatory microglia/macrophage load in peri-stroke areas. Our data might contribute to the explanation of neutral and negative results of clinical trials using autologous BM-MNC transplantation for ischemic diseases, especially transplantation of cells isolated by automatic cell separators such as the Sepax2.

BM-MNC therapy has been initiated for limb ischemia and in ischemic wound healing with angiographic findings and physiological parameters being reported.[23] However, non-responders to cell therapy exist[24,25] and both positive[26,27] and neutral/negative[28,29] results have been reported in double blinded, placebo-controlled studies using autologous BM-MNC transplantation for myocardial infarction. The reasons of the contradictory results are currently unclear. BM aspiration fluid contains various cells/substances, including connective tissue, bone fragments, and lipids. These substances trigger the coagulation cascade and clot formation is observed to a greater or lesser extent in many cases.[30] Our results show that transplantation of clot-derived contaminants augments microglia/macrophage numbers in peri-stroke areas and impairs the effect of cellular therapy, although transplanted BM-MNC still preserved the microvasculature in the lesion area. Our results could help to explain neutral and negative

results in clinical trial using Sepax2-isolated BM-MNC transplantation in myocardial ischemia,[31] which are in contrast to the positive results after using manually isolated BM-MNC.[32]

Intra-arterial cell delivery is gaining momentum based on technical improvements and widespread application of thrombectomy. Intra-arterial cell transplantation has a significant advantage of delivering more cells to where they are exactly needed, avoiding peripheral filtering effects, for instance in lungs. However, the therapeutic effects of intra-arterial BM-MNC had been shown to be not superior to intravenous transplantation in experimental stroke.[18,33] Moreover, intra-arterial transplantation would delivers higher amounts of clot-derived contaminants directly to the brain if cells are not optimally prepared, potentially offsetting additional therapeutic benefits gained by the approach. This could explain the reason why intra-arterial BM-MNC is not superior to intravenous transplantation and indicated the quality of cell preparation may particularly matter for the selection of delivery route.

Positive effects of thrombectomy for stroke patients are increasingly reported. Significant benefits of clot removal up to 24 h after stroke onset was shown[34] and the effect of clot removal beyond 24 h was also suggested.[35] Our current results regarding enhanced presence of proinflammatory microglia/macrophages by clot derived substances indicates that thrombectomy would be not only important for blood flow restoration, but would also mitigate clot-borne neuroinflammation.

Microglia play an important role in post-stroke pathophysiology, but discrimination of microglia and macrophages is challenging.[36] The origin of activated microglia/macrophage by clot-derived contaminants in peri-stroke areas can be local microglia, clot-derived cells, or circulating macrophages. In this study, many F4/80-positive microglia/macrophages in peri-stroke were CMFDA-/Alexa 647-positive in mice that received clot-derived cells/debris. Since tissue was obtained as early as 20 minutes after stroke induction, we speculate that the origin of activated microglia/macrophage within peri-stroke areas is not an enhanced ingress of activated macrophages, but enhanced activation of local microglia.

In conclusion, our results indicate that the exclusion of clot-derived contaminants in transplanted bone marrow mononuclear cells is a critical factors for BMM-MNC therapy and can explain neutral or negative results in clinical trials using automatic cell separators which do not have the ability to remove clots from the buffy coat. Our data also emphasizes the importance of the clarification of the therapeutic mechanisms for the improvement of therapeutic effect of cell therapy.

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Disclosures

None.

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Figure legends

Figure 1. Design of *in vivo* experiments

Schematic illustration of *in vivo* experimental design.

Figure 2. Isolated human BM-MNC

(A) In the process of manual cell isolation, clots (arrows) were observed at the buffy coat after gravity centrifugation. (B, C) Compared to manually isolated BM-MNC, Sepax2 isolated BM-MNC and cells in clots contain significantly much more RBC and platelets/cell debris. Red and black arrow heads indicate RBC and platelets/cell debris, respectively. Scale bars, 1 cm (A) and 10 μm (B). *p<0.01 vs manual, #p<0.01 vs Sepax2, N=6 views in each group (C).

Figure 3. Sepax2 isolated BM-MNC did not show therapeutic effect after stroke

(A) Brain images 30 days after stroke induction. (B) The areas of left and right hemisphere (HS) were measured. Compared to control, significant attenuation of brain atrophy was observed only in mice that received manually processed BM-MNC. Scale bar, 2.5 mm (A). *p<0.01, N=9 in each group (B).

Figure 4. Activation of microglia/macrophage after Sepax2 isolated cell transplantation

(A) Schematic illustration of lesion and peri-stroke area. (B) Mice that received PBS showed

microvascular narrowing, indicating vessel breakdown in the lesion area. In contrast, microvasculature was preserved in mice that received manually or Sepax2-isolated BM-MNC. (C, D) Mice that received PBS and manually isolated cells showed mild activation of CD11b-positive microglia/macrophages in peri-stroke areas. In contrast, transplantation of Sepax2-isolated BM-MNC was associated with higher microglia/macrophage numbers. Scale bars, 100 μm (B, C; upper panel), 50 μm (B, C; lower panel). *p<0.01 versus manual, N=6 in each group (D).

Figure 5. Injection of clot-derived cells/debris augments microglia/macrophage activation at peri-stroke area

(A) No F4/80-positive microglia/macrophages with incorporated CMFDA or Alexa 647 were observed in mice that received cells before clot formation. (B) In contrast, microglia/macrophages incorporating CMFDA and Alexa 647 were observed when transplanting clot-derived, labelled cells/debris. Upper and lower panels show lower and higher magnification, respectively. Scale bars, 20 μm (A; upper panel), 10 μm (A; lower panel).

Figure. 1

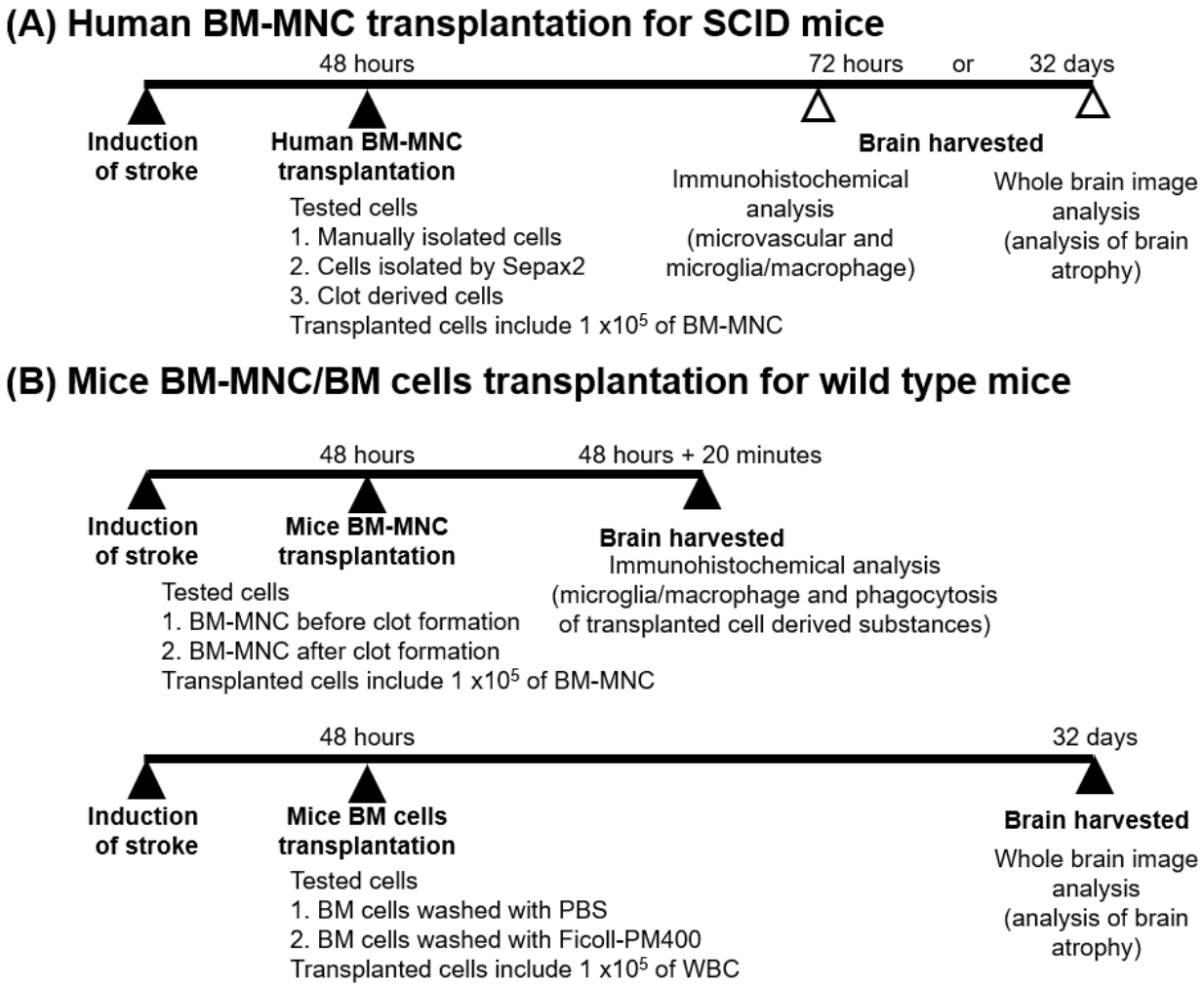

(A) Human BM-MNC transplantation for SCID mice
48 hours
72 hours
or
32 days
Induction of stroke
Human BM-MNC transplantation
Brain harvested
Immunohistochemical analysis (microvascular and microglia/macrophage)
Whole brain image analysis (analysis of brain atrophy)
Tested cells
1. Manually isolated cells
2. Cells isolated by Sepax2
3. Clot derived cells
Transplanted cells include 1 x10^5 of BM-MNC
(B) Mice BM-MNC/BM cells transplantation for wild type mice
48 hours
48 hours + 20 minutes
Induction of stroke
Mice BM-MNC transplantation
Brain harvested
Immunohistochemical analysis (microglia/macrophage and phagocytosis of transplanted cell derived substances)
Tested cells
1. BM-MNC before clot formation
2. BM-MNC after clot formation
Transplanted cells include 1 x10^5 of BM-MNC
48 hours
32 days
Induction of stroke
Mice BM cells transplantation
Brain harvested
Whole brain image analysis (analysis of brain atrophy)
Tested cells
1. BM cells washed with PBS
2. BM cells washed with Ficoll-PM400
Transplanted cells include 1 x10^5 of WBC

Figure. 2

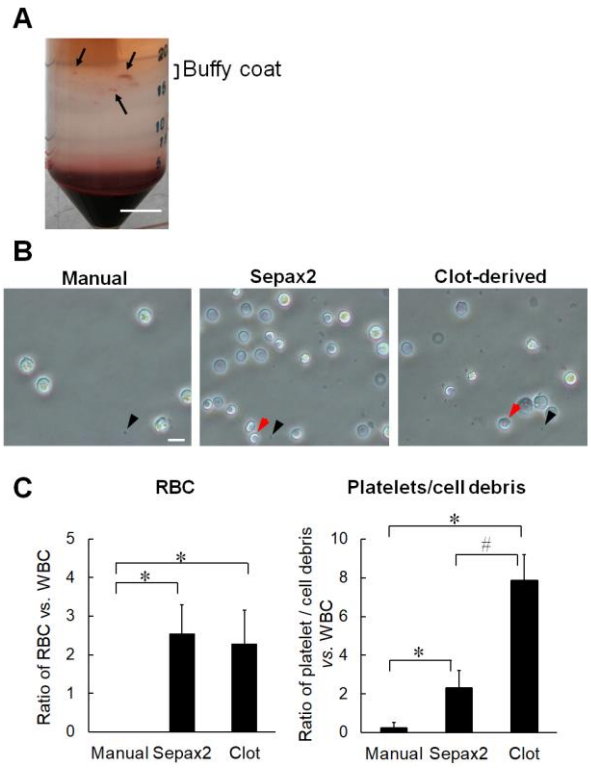

A
Buffy coat
B
Manual
Sepax2
Clot-derived
C
RBC
Platelets/cell debris
Ratio of RBC vs. WBC
Ratio of platelet / cell debris vs. WBC
Manual Sepax2 Clot
Manual Sepax2 Clot

Figure. 3

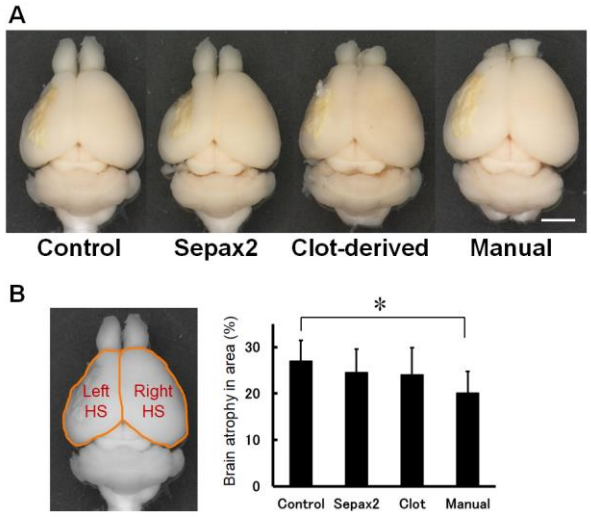

A
Control
Sepax2
Clot-derived
Manual
B
Left HS
Right HS
Brain atrophy in area (%)
30
20
10
0
*
Control
Sepax2
Clot
Manual

Figure. 4

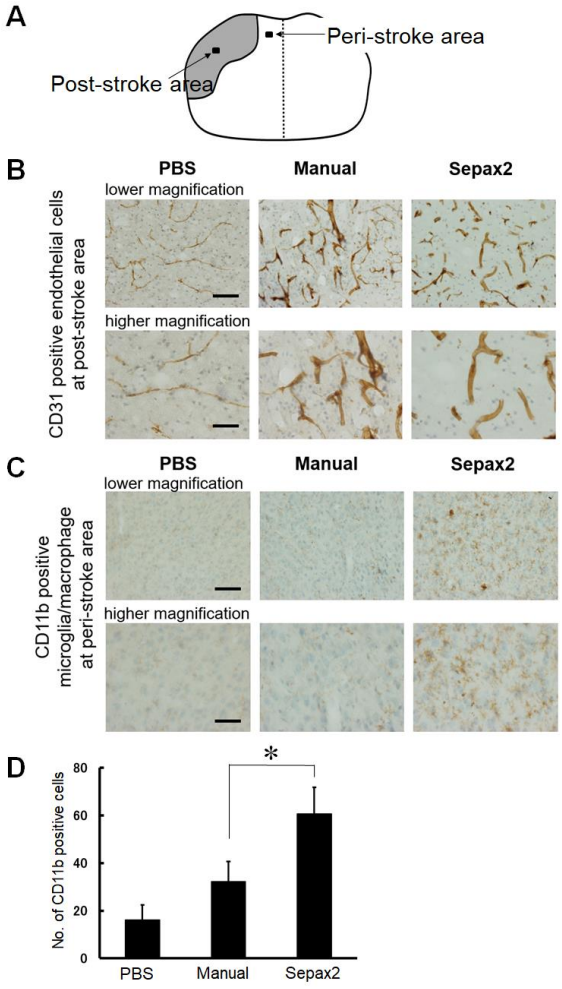
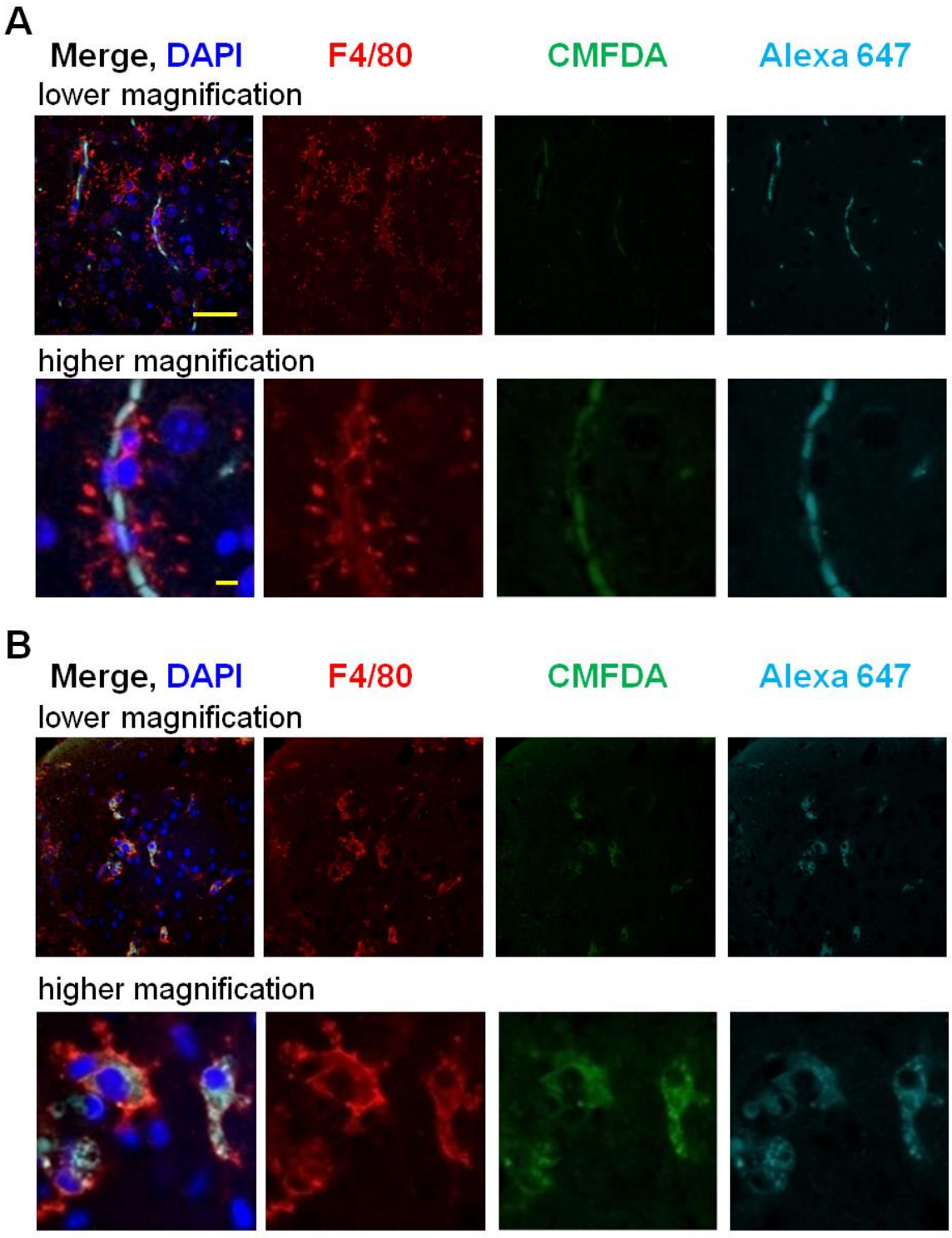

A
Peri-stroke area
Post-stroke area
B
PBS
Manual
Sepax2
lower magnification
higher magnification
CD31 positive endothelial cells at post-stroke area
C
PBS
Manual
Sepax2
lower magnification
higher magnification
CD11b positive microglia/macrophage at peri-stroke area
D
*
No. of CD11b positive cells
80
60
40
20
0
PBS
Manual
Sepax2

Figure. 5


A
Merge, DAPI
F4/80
CMFDA
Alexa 647
lower magnification
higher magnification
B
Merge, DAPI
F4/80
CMFDA
Alexa 647
lower magnification
higher magnification